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Running title: SNPs for genetic characterization of Italian beef cattle

Comparison of four Italian beef cattle breeds by means of functional genes

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20 **Abstract**

21 Piemontese, Chianina, Marchigiana and Romagnola are the main Italian beef breeds, and the quality
22 of their products is largest recognized all over the world. Here, 18 SNPs in 12 candidate genes
23 involved on meat traits were investigated on 1055 candidates for selection in order to analyze the
24 within and between breed variability with a functional marker approach.

25 Three SNPs (GDF8-3, GH and NPY-3) were monomorphic and most of the polymorphic SNPs
26 showed an allele distribution quite similar in the four breeds. High variability at LEP-2, LEP-3 and
27 LEPR markers was detected across breed and the analysis of the relationship between F_{ST} and
28 heterozygosity suggested a different selection intensity by breeds for LEP-2. The highest pairwise
29 F_{ST} values (0.1189 to 0.1877) were obtained for the comparisons of Piemontese with the other
30 breeds, while the lowest value (0.0296) was observed between Chianina and Marchigiana. The
31 Piemontese differentiation from the other breeds could be due to its geographical isolation and
32 selection targets. The results for breed assignment follows the genetic differentiation, in fact,
33 Piemontese had the highest percentage of correct assignment (87.6), while Marchigiana had the
34 lowest one (47.5). These findings suggest that the functional markers can be more suitable than
35 neutral markers in discriminating breeds in similar morphology if selection played some role in
36 their differentiation.

37
38 **Keywords:** Chianina, Marchigiana, Piemontese, Romagnola, SNPs

39
40 **Introduction**

41 The Italian beef cattle breeds have always been connected with rural and ethnic traditions, therefore
42 they represent a historical and cultural heritage which exceeds their economic value. Among them,
43 Piemontese, Chianina, Marchigiana and Romagnola are the main specialized breeds for meat
44 production and the quality of their products is widely recognized all over the world.

45 Several studies focused on the genetic description of these breeds and their relationships. For
46 example, on the basis of biochemical markers, Baker and Manwell (1980) included Chianina,
47 Marchigiana and Romagnola in the Italian podolic group belonging to the *Primigenius* taxon, while
48 Piemontese was included in the *Primigenius-brachyceros* Mixed taxon. Concordant results on the
49 four studied breed grouping were obtained by Blott *et al.* (1998), using blood groups and protein
50 polymorphisms. More recently, molecular markers, such as AFLP (Negri *et al.*, 2007) and
51 microsatellites (Dalvit *et al.*, 2008), were used to characterize the same breeds in the framework of
52 product traceability.

53 The latter two studies were based on neutral markers, which are routinely used to analyse the
54 genetic structuring of populations, being the most effective in detecting the relationships among
55 breeds determined by processes such as migration and genetic drift. However, there is a growing
56 evidence that variation in functional sequences can be more efficient in highlighting differences
57 among breeds induced by selection (van Tienderen *et al.*, 2002; Kirk and Freeland, 2011,
58 Pampoulie *et al.*, 2011).

59 The breeds here considered are all beef breeds, but the selection programmes implemented by the
60 respective National Breeders' Associations in the course of time are quite different (Albera *et al.*,
61 2001; Sbarra *et al.*, 2009). At present the emphasis of the selection in the Piemontese breed is on
62 reducing calving problems, while improving growth rate and meat conformation (ANABORAPI,
63 2013). For Chianina, Marchigiana and Romagnola the selection has always been focused on
64 improving daily gain and muscle conformation (ANABIC, 2013).

65 As many candidate genes have been suggested for their potential effects on meat traits (Li *et al.*,
66 2004; Buchanan *et al.*, 2005; Nkrumah *et al.*, 2005; Di Stasio *et al.*, 2007; Sherman *et al.*, 2008),
67 the present investigation was carried out in order to analyze the within and between breed
68 variability in Chianina, Marchigiana, Piemontese and Romagnola breeds with a functional marker
69 approach.

71 **Material and methods**

72 *Animal sampling and molecular analysis*

73 Blood samples were collected from a total of 1055 candidates evaluated using a performance
74 testing: 359 Chianina (CHI), 242 Marchigiana (MAR), 226 Piemontese (PIE) and 228 Romagnola
75 (ROM). Genomic DNA was extracted from blood using the GenElute Blood Genomic DNA kit
76 (Sigma Aldrich, St. Louis, MO, USA).

77 According to a preliminary bibliographic survey, 18 SNPs in the following 12 genes were selected
78 on the basis of the reported correlations with beef traits: *growth hormone* (GH), *growth hormone*
79 *receptor* (GHR), *growth differentiation factor 8* (GDF8), *ghrelin* (GHRL), *leptin* (LEP), *myogenic*
80 *factor 5* (MYF5), *insulin-like growth factor 2* (IGF2), *leptin receptor* (LEPR), *neuropeptide Y*
81 (NPY), *proopiomelanocortin* (POMC), *uncoupling protein 2* (UCP2) and *uncoupling protein 3*
82 (UCP3). The list of the studied SNPs is reported in Table 1.

83 The genotyping of the investigated SNPs was performed by LGC Genomics (Hoddesdon, Herts,
84 UK) using KASPar technology. To assess the genotyping accuracy, 10% of the samples were
85 genotyped in duplicates.

86

87 *Statistical analysis*

88 The allele frequencies, observed and expected heterozygosity were calculated by the FSTAT
89 software version 2.9.3.2 (Goudet, 2002). F_{IS} per breed across loci was calculated using the software
90 GENETIX version 4.05 (Belkhir *et al.*, 1996-2004), while single-locus F_{ST} , pairwise F_{ST} and global
91 F_{ST} were estimated using FSTAT software version 2.9.3.2 (Goudet, 2002). The FDIST2 program
92 (Beaumont and Nichols, 1996) was used to test loci for selective neutrality under an infinite alleles
93 mutational model. The linkage disequilibrium between SNPs was tested by the software GENETOP
94 4.0 (Raymond and Rousset, 1995), using Bonferroni correction. For the linked SNPs, the haplotype
95 frequencies were estimated by the software PHASE version 2.1 (Stephens and Scheet, 2005). The
96 percentage of correct assignment per breed was calculated by the GeneClass2 software (Piry *et al.*,

2004), using the distance method, which does not require the assumption of independence among loci. Of the different genetic distance option, the D_a (Nei *et al.*, 1983) was used. The assignment was considered correct when the probability was higher than 50%. For each breed the assignment of 20 individuals not in the reference sample was also tested.

101

102 **Results and discussion**

103 Three SNPs (GDF8-3, GH and NPY-3) were monomorphic in all the breeds (Table 2). The finding
104 is not surprising for GH and NPY-3, which were reported to be polymorphic only in one or few
105 breeds (Kim *et al.*, 2004; Sherman *et al.*, 2008), while it was unexpected for GDF8-3, for which
106 polymorphism had been described in the Piemontese breed, though in a more limited sample
107 (Vankan *et al.*, 2010). It is also interesting to note that in the Piemontese GDF8-1 was
108 monomorphic too, while variability was reported by Crisà *et al.* (2003) in the same breed.

109 For most of the polymorphic SNPs, the allele distribution was quite similar in the four breeds, with
110 the predominance of the same allele. The main differences concerned LEP-2, LEP-3 and LEPR loci.
111 For seven SNPs (GHR-2, GHRL, IGF2, NPY-1, NPY-2, UCP-2 and UCP-3) the observed
112 frequencies are in the range reported by Sherman *et al.* (2008) for European beef cattle breeds.

113 The variability of the single loci across breed, estimated by F_{ST} , showed a wide range, between
114 0.005 (GHR-3) and 0.238 (LEP-2). High levels of genetic divergence were also observed for LEP-3
115 (0.204) and, to a lesser extent, LEPR (0.159). It has been shown that F_{ST} values can help in
116 detecting markers under directional selection or experiencing different strength of selection,
117 because they are expected to show higher differentiation across breeds than neutral loci (Beaumont
118 and Nichols, 1996; Narum and Hess, 2011). The distribution of F_{ST} as a function of heterozygosity
119 indicated that all the markers, except for LEP-2, fall within the 0.95 limits (Figure 1). This finding
120 suggests for LEP-2 deviations from a neutral-equilibrium model, possibly due to selection acting
121 with different intensity in different breeds.

122 The heterozygosity values at single loci (data not shown) differed between breeds according to the
 123 allele frequencies, but the overall values were very similar. The F_{IS} values were not significant,
 124 indicating a low level of inbreeding in the four breeds (Table 3).

125 A significant ($P = 0.0005$) linkage disequilibrium was observed only for the SNPs located in the
 126 same gene: GHR-1 - GHR-2, LEP-1 - LEP-2 - LEP-3, NPY-1 - NPY-2.

127 The haplotypes frequencies (Table 4) showed a quite different situation across breeds. For example,
 128 Romagnola differed from the other breeds for the most frequent haplotype at GHR and NPY loci.
 129 For LEP gene, a total of 8 haplotypes were observed, with CCT more frequent, except for
 130 Piemontese. Some of the rarest haplotypes were absent in a given breed: TCC in Chianina, CGT
 131 and TGT in Marchigiana, TCT in Piemontese.

132 The genetic differentiation (F_{ST}) in the overall sample (Table 5) was high (0.085; $P=0.001$) with
 133 respect to the value of 0.049 obtained in a comparable study on the same breeds using microsatellite
 134 markers (Dalvit *et al.*, 2008). The pairwise F_{ST} also detected a higher degree of between breed
 135 variability, so that the functional markers seemed to be even more valuable than neutral markers in
 136 detecting variability among these breeds. The picture of the relationships among breeds was also
 137 different from the one shown by neutral markers. In fact, the highest pairwise F_{ST} values (0.1189 to
 138 0.1877) were obtained in the comparisons of Piemontese with the other breeds, while the lowest
 139 value (0.0296) was observed between Chianina and Marchigiana. The differentiation of Piemontese
 140 from the others three breeds, already observed with different markers (Ciampolini *et al.*, 1995; Blott
 141 *et al.*, 1998), supports the phylogenetic origin described by Baker and Manwell (1980). Moreover,
 142 the geographical isolation of the Piemontese and, more recently, the difference in selection indexes
 143 could have contributed to its differentiation. The higher similarity among the breeds of the Central
 144 Italy is consistent with both their known history and common selection programmes. In particular,
 145 the closeness of Marchigiana with Romagnola and especially Chianina is expected on the basis of
 146 its documented origin from crossing of local Marche cattle with the two breeds (Bonadonna, 1976).

147 The results for breed assignment reflected the genetic differentiation of the breeds (Table 6). In
148 agreement with data reported in different studies with different breeds and markers (Ciampolini *et*
149 *al.*, 2000; Negrini *et al.*, 2007; Dalvit *et al.*, 2008), the Piemontese breed had the highest percentage
150 of correct assignment (87.6, with 61% of the values exceeding 95%), while Marchigiana had the
151 lowest one (47.5, with only 4% of the values exceeding 95%). Moreover, the wrongly assigned
152 Marchigiana animals were mainly classified as Chianina because of their low genetic differentiation
153 ($F_{ST} = 0.03$).

154 The assignment test of independent samples confirmed the best results for the Piemontese breed,
155 with 19 out of 20 animals correctly assigned. For the other breeds, in the same test, the percentage
156 of correct assignment ranged from 55% for Romagnola to 70% for Chianina.

157

158 **Conclusions**

159 The results showed that for the breeds here considered functional markers allowed to detect a
160 greater level of genetic differentiation compared to that observed for the same breeds with neutral
161 markers. The two classes of markers reflect between-breed differences due to different sources of
162 variation, mainly genetic drift for neutral markers and selection for functional markers. Therefore,
163 in a more general view, the combined study of neutral markers and SNPs in functional regions can
164 provide complementary information about the genetic dynamics of the breeds within a species.

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166 **Acknowledgements**

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168 The authors want to thank the two anonymous referees for their valuable comments and
169 constructive suggestions.

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Table 1. Information on the studied SNPs.

Gene	Chromosome	SNP name	Location	Accession No and base position	SNP
GH	BTA19	GH	Promoter	AY445811:g.358	C>T
GHR	BTA20	GHR-2	Promoter	AF126288:g.149	G>A
GHR	BTA20	GHR-3	Intron IV	AY643807:g.300	A>G
GDF8	BTA2	GDF8-1	Promoter	AJ438578:g.843	T>A
GDF8	BTA2	GDF8-3	Exon I	AY725215:g.229	A>C
GHRL	BTA22	GHRL	Intron III	AY455980:g.446	A>G
LEP	BTA4	LEP-1	Promoter	AB070368:g.528	C>T
LEP	BTA4	LEP-2	Promoter	AB070368:g.1759	G>C
LEP	BTA4	LEP-3	Exon II	AY138588:g.305	T>C
MYF5	BTA5	MYF5	Intron II	M95684:g.1948	A>G
IGF2	BTA29	IGF2	Exon II	AY237543:g.150	C>T
LEPR	BTA3	LEPR	Exon XX	AJ580801:g.115	C>T
NPY	BTA14	NPY-1	Intron II	AY491054:g.284	A>G
NPY	BTA4	NPY-2	Intron II	AY491054:g.666	A>G
NPY	BTA4	NPY-3	Intron II	AY491054:g.3032	C>T
POMC	BTA11	POMC	Intron II	J00021:g.254	C>T
UCP2	BTA15	UCP2	Intron V	AY14782:g.380	G>C
UCP3	BTA15	UCP3	Intron III	AF127030:g.1099	G>A

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Table 2. Alleles frequencies in the studied SNPs (only one allele per SNP is reported).

SNP name	Alleles	Breeds				F_{ST}
		CHI	MAR	PIE	ROM	
GDF8-1	A	0.247	0.171	0.000	0.099	0.074
GDF8-3	C	1.000	1.000	1.000	1.000	-
GH	C	1.000	1.000	1.000	1.000	-
GHR-2	A	0.496	0.620	0.462	0.215	0.087
GHR-3	A	0.752	0.682	0.665	0.720	0.005
GHRL	A	0.857	0.932	0.797	0.952	0.037
IGF2	C	0.787	0.669	0.749	0.765	0.010
LEP-1	C	0.937	0.833	0.597	0.633	0.105
LEP-2	C	0.781	0.633	0.137	0.399	0.238
LEP-3	C	0.210	0.407	0.830	0.541	0.204
LEPR	C	0.563	0.529	0.926	0.403	0.159
MYF5	A	0.416	0.560	0.426	0.424	0.014
NPY-1	A	0.097	0.060	0.232	0.129	0.036
NPY-2	C	0.267	0.178	0.311	0.491	0.061
NPY-3	A	1.000	1.000	1.000	1.000	-
POMC	C	0.802	0.924	0.819	0.956	0.039
UCP2	C	0.930	0.917	0.810	0.853	0.022
UCP3	A	0.625	0.581	0.774	0.426	0.064

CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.

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Table 3. Mean observed heterozygosity (H_o), mean expected heterozygosity (H_e) and F_{IS} in the studied breeds.

Breeds	H_o	H_e	F_{IS}
CHI	0.35 (0.13)	0.34 (0.13)	-0.027 (-0.057 – 0.001)
MAR	0.34 (0.159)	0.34 (0.15)	0.005 (-0.039 - 0.043)
PIE	0.34 (0.14)	0.33 (0.14)	-0.022 (-0.061 - 0.013)
ROM	0.36 (0.16)	0.36 (0.15)	-0.008 (-0.051 - 0.029)

187 CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.

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Table 4. Haplotype frequencies.

Gene	Haplotype	Breeds			
		CHI	MAR	PIE	ROM
GHR	[GHR-2, GHR-3]				
	AA	0.49574	0.61981	0.43393	0.20685
	AG	0.00004	0.00002	0.02708	0.01064
	GA	0.25426	0.06200	0.22891	0.51288
	GG	0.24996	0.31816	0.31008	0.26963
LEP	[LEP-1, LEP-2, LEP-3]				
	CCC	0.03602	0.03427	0.00485	0.00493
	CCT	0.74430	0.59319	0.13779	0.38868
	CGC	0.11462	0.20449	0.45569	0.24038
	CGT	0.04133	0.00000	0.00020	0.00045
	TCC	0.00000	0.00515	0.00022	0.00014
	TCT	0.00014	0.00018	0.00000	0.00012
	TGC	0.06183	0.16273	0.36683	0.29229
	TGT	0.00178	0.00000	0.03443	0.07302
NPY	[NPY-1, NPY-2]				
	AC	0.00034	0.00037	0.00025	0.00020
	AT	0.09715	0.05955	0.23017	0.12760
	GC	0.26707	0.17731	0.30851	0.49307
	GT	0.63544	0.76277	0.46108	0.37913

CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.

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197 **Table 5.** Pairwise and global F_{ST} .
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	CHI	MAR	PIE	ROM
CHI	-			
MAR	0.0296	-		
PIE	0.1877	0.1403	-	
ROM	0.1029	0.0786	0.1189	-
Global F_{ST}	0.0848 ($P = 0.001$)			

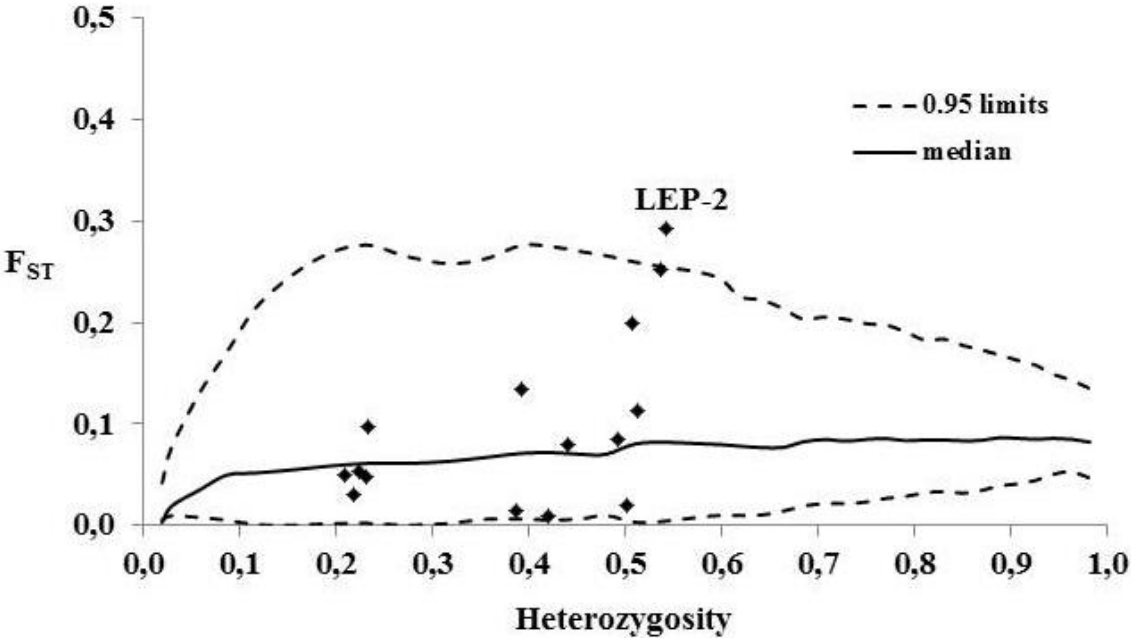
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 200 CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.
 201 After Bonferroni's correction all the values are significant.
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205 **Table 6.** Percentage of animals assigned to each breed.
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Breeds	Assigned to				Mean probability of assignment
	CHI	MAR	PIE	ROM	
CHI	70.8	15.9	5.8	7.5	79.3
MAR	31.4	47.5	10.8	10.3	69.5
PIE	3.5	5.3	87.6	3.6	91.0
ROM	15.4	7.9	11.8	64.9	82.9

207 CHI: Chianina, MAR: Marchigiana, PIE: Piemontese ROM: Romagnola.
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210 Figure 1. F_{ST} values estimated for the 15 polymorphic markers, plotted against heterozygosity.
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